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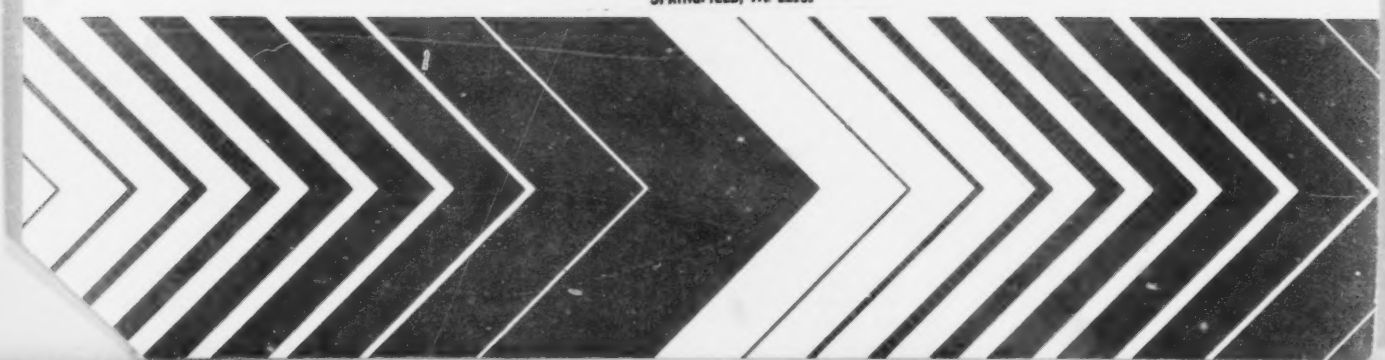

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16. ABSTRACT

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EFFECTS OF PESTICIDES ON THE IMMUNE RESPONSE

by

Walter B. Dandliker, Arthur N. Hicks,
Stuart A. Levison, Kris Stewart,
and R. James Brawn
Department of Biochemistry
Scripps Clinic and Research Foundation
La Jolla, CA 92037

Grant No. R803885

Project Officer

August Curley
Environmental Toxicology Division
Health Effects Research Laboratory
Research Triangle Park, NC 27711

HEALTH EFFECTS RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
RESEARCH TRIANGLE PARK, NC 27711

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TABLE 1. Effects of Pesticides on the Immune Response as shown by Parameters Pertaining to Serum Antibody

Pesticide	Dose of Immunogen	Days	P_b	Q_f/Q_b	10^{-8} K_o	a	$10^9 F_{b,max}$	X^2
Control	L	31	0.48	7.1	5.0	0.75	0.90	1.38
"	"	46	0.45	5.0	0.9	0.77	4.5	1.49
"	"	52	0.42	3.1	3.8	0.77	6.5	1.78
Control	H	46	0.40	4.0	2.0	1	0.86	1.06
"	"	52	0.40	4.0	0.20	0.85	4.7	0.37
Methotrexate	H	46	0.36	6.2	0.77	0.85	9.9	0.47
"	"	52	0.42	5.9	0.76	0.77	5.1	1.86
Aroclor	L	29	0.43	6.4	3.1	0.70	5.4	1.11
"	"	36	0.44	4.5	3.3	0.72	6.3	0.76
Dinoseb	L	49	0.42	5.0	2.3	0.93	1.11	1.02
Parathion	L	42	0.45	5.8	6.6	1.0	0.41	1.41
"	"	49	0.45	4.4	1.4	0.77	1.3	0.69
PCNB	H	46	0.42	5.8	1.00	0.87	4.1	2.49
"	"	52	0.40	8.6	1.15	0.83	4.0	0.42
Pip. Butox	L	29	0.45	6.4	2.2	0.76	5.0	1.23
"	"	36	0.45	6.6	5.1	0.77	6.7	1.19
Pyrethrins	H	46	0.40	5.9	0.67	0.91	2.0	0.35
"	"	52	0.42	4.7	0.35	0.89	3.5	0.76
Resmethrin	L	29	0.46	5.8	5.5	0.80	4.7	1.17
"	"	36	0.47	5.0	10.1	0.83	9.0	1.24

L = 0.4 mg immunogen/hamster

H = 4 mg immunogen/hamster

P_b, the polarization of bound fluorescein

Q_f/Q_b, the fluorescence ratio of free to bound

K_o, the association constant, $1mole^{-1}$

a, the heterogenecity index

$F_{b,max}$, the antibody site concentration in 300 X diluted serum

$X^2 = 100\ \Sigma \left(P_{obs} - P_{calc}\right)^2$, statistical measure of fit between observed and calculated polarization

FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardsous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This report summarizes the results of a study to determine the effects of pesticides on immune responses. Immediate toxic effects are relatively readily assessed but slow or delayed effects are more difficult to detect and yet may be more important -- possibly leading to altered susceptibility to disease, damage in utero, accelerated aging, tumorgenesis, etc. Immune responses, although very little studied, offers one parameter which is a sensitive indicator of a variety of physiological functions and which may be quantitated. The results of this study offers methodology useful in assessing the adverse effects of pesticides exposure on immune response.

F. G. Hueter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

The influence of various pesticides on the humoral and cellular immune response to fluorescein labeled ovalbumin has been analyzed. Pesticides (Aroclor 1260, Dinoseb, Parathion, pentachloronitrobenzene, piperonyl butoxide, mixed pyrethrins and Resmethrin) were administered intragastrically in corn oil in one dose (one half of LD_{50}) before primary immunization. Control groups included those treated with corn oil alone or immunosuppressed with Methotrexate. Booster immunizations and test bleedings were scheduled at weekly intervals thereafter. The cellular immune response was quantified by redness and swelling, histological examination and by differential temperature measurements of the foot pads after antigen challenge. The concentration, binding affinity and heterogeneity of the serum antibody were determined by fluorescence polarization measurements. Dinoseb and Parathion depress both the humoral and cellular response. Methotrexate and pentachloronitrobenzene give a late stimulation, while Resmethrin an early, sometimes very marked stimulation of the cellular immune response. Other pesticides showed little or no effect under the conditions tested. Effects on the humoral response were limited to changes in antibody concentration, the binding affinity being nearly constant in all instances.

TABLE OF CONTENTS

Foreword iii

Abstract iv

Introduction 1

Materials & Methods 3

Results 6

Discussion 9

References 13

Appendix 23

Introduction

The world-wide use of pesticides makes it urgent to know as much as possible about the effects of pesticides and their degradation products on humans and animals. Immediate toxic effects are relatively readily assessed but slow or delayed effects are more difficult to detect and yet may be the more important -- possibly leading to altered susceptibility to disease, damage in utero, accelerated aging, etc.

The immune response offers one parameter which is a sensitive indicator of a variety of physiological functions and which is readily quantified in a number of ways. The early work in this area was reviewed by Ercegovich (1). Since that time there has been a growing interest in evaluating pesticide effects on a number of different aspects of the immune response. These include effects on antibody production, dermal reactions to specific immunogens, immunoglobulin levels, resistance to infection, complement levels, lymphoid cell counts and effects on lymphoid organs detected histologically.

The parameters most easily quantified are those connected with the humoral response since serum antibody can be easily obtained and can be characterized in a number of ways. The production of anti-human serum albumin was found to be inhibited in rats injected with Lindane, 60 or 120 mg/kg (2), while the titer of anti-bovine serum albumin was not consistently altered in chicks fed mash containing up to 625 parts per million (ppm) of DDT (3). Hemagglutinin levels of rats immunized against sheep red blood cells were suppressed by an oral dose of Methylnitrophos or Chlorophos,

5 or 7 mg/kg/day, especially in rats fed a protein-deficient diet (4). A second class of specific immunogen used to monitor the humoral immune response in pesticide-treated animals includes bacteria and viruses. Significantly lowered titers of anti-Salmonella typhii were found in rabbits given drinking water containing 200 ppm DDT (5-7). Also the expected increase in the γ-globulin 7S fraction in response to Salmonella inoculation was inhibited by Dieldrin and benzene hexachloride (8). On the other hand, no consistent effect was found in the anti-Salmonella pullorum titer of DDT-fed chicks (3). Similarly, no differences in bacterial agglutination, indirect hemagglutination, indirect hemolysis, or precipitation were noted between Warfarin-treated and untreated rabbits immunized with purified Salmonella typhii endotoxin (9). Lower titers of tetanus antitoxin were consistently found when animals immunized with tetanus toxoid were given dietary Aroclor 1260 or Clophen A60 (50 ppm) (10) or 0.1 - 0.2 LD_{50} of Carbaryl orally (11). Effects on antibody-mediated immunity have been investigated for Anthio and Milbex in goats (12,13) and for Minex and DDT in chickens (14). Examination of lymphoid organs proved to be a sensitive indicator of immunosuppression in a study of the effects of DDT, Aroclor 1254, Carbaryl, Carbofuran and Methylparathion in rabbits (15).

The present study was designed to simultaneously analyze the influence of various pesticides on the humoral and cellular immune responses to a well defined immunogen (fluorescein labeled ovalbumin). The pesticides were administered in one oral dose preceding primary immunization. Booster immunizations were then given periodically

after sampling the serum and performing *in vivo* tests of cellular immunity. These included visual evaluation of redness and swelling of a challenged footpad and measurement of the temperature difference between challenged and control footpads. Serum antibody was characterized by fluorescence polarization. In addition, body weight was followed as an indicator of gross physiological status throughout the experiments.

Materials and Methods

Pesticides. Dinoseb, Parathion and Pentachloronitrobenzene were analytical standards (16) from the Environmental Protection Agency, Triangle Park, N.C. Resmethrin, piperonyl butoxide and mixed natural pyrethrins were generously donated by FMC Corporation, Agricultural Chemicals Group, Richmond, CA, and Aroclor 1260 was purchased from Chem Service (West Chester, PA). Methotrexate was the pharmaceutical product from Lederle Laboratories (Pearl River, N.Y.).

Animals. Inbred male hamsters, Strain LHC/LAK (Lakeview Hamster Colony, Newfield, NJ) 5 to 8 weeks old and weighing about 100 g each were used for all experiments.

Preparation of Immunogen. To 5 g of chicken ovalbumin (Mann Research Laboratories, New York, NY, 5X crystallized) dissolved in 15 ml of 0.5 M carbonate buffer (0.4 M $NaHCO_3$, 0.1 M Na_2CO_3, pH 9.3), 43 mg fluorescein isothiocyanate (Isomer I, Sigma Chemical Co., St. Louis, MO) was added. The mixture was held at 4° for 16 hr and passed through a column (3.7 cm in diameter X 58 cm long) of Sephadex G-25 medium (Pharmacia Inc., Piscataway, NJ) previously equilibrated with 0.15 M NaCl. The void volume as determined by

blue dextran was 250 ml. The fluorescein labeled ovalbumin (FO) was collected in a volume of 143 ml beginning at 246 ml of effluent. Optical density measurements at 280 and 490 nm indicated a labeling ratio of 1.0 utilizing the molar extinction coefficients for fluorescein previously determined (17) and assuming values for ovalbumin of 7.34 for $E^{1\%}_{280}$ and of 46,000 for the molecular weight. The FO solution was stored at -20° until used.

To prepare FO for injection, a solution containing 20, 2 or 0.4 mg/ml (in 0.15 M NaCl) was homogenized with an equal volume of Complete Freund's Adjuvant (CFA) (DIFCO Laboratories, Detroit, MI) by means of two syringes connected together by a plastic 3-way stopcock (Type K-75, Pharmaseal, Toa Alta, Puerto Rico).

Pesticide Administration. Each animal received one dose of pesticide or other compound equal to one-half of the LD_{50} (16) dissolved in 1 ml of corn oil. The pesticide solution was administered as a bolus by intragastric feeding tube 24 hr after the first injection of FO. The animals were fasted during this 24 hr period with water *ad lib*.

Immunization. For all immunizations, 0.2 ml of a mixture consisting of equal volumes of FO and CFA were injected subcutaneously into each flank. For primary immunizations FO at either 10 mg/ml or 1 mg/ml (final concentration in homogenized mixture) was used resulting in a dose of either 4 mg or 0.4 mg per hamster. Booster immunizations consisted of 0.2 ml of FO-CFA mixture containing 200 µg FO/ml into each flank. The primary immunization was given 24 hr after the pesticide; boosters were administered at 7 day intervals after the primary immunization.

Serum preparation. All blood was drawn by cardiac puncture, allowed to clot 2 hr at room temperature, overnight at +4° and the serum drawn off after centrifugation. A preimmunization bleeding was obtained before pesticide administration and thereafter before each booster immunization.

Immunoglobulin preparation. Serum pooled from several individual bleedings was fractionated by ammonium sulfate precipitation to give an immunoglobulin preparation substantially free of serum albumin (which if present in sufficient concentration, would interfere in the titrations by binding fluorescein non-specifically). To one volume of serum, 0.58 volumes of saturated ammonium sulfate (adjusted to pH 8.1 to 8.2 by the addition of concentrated NH_4OH) was added rapidly while mixing, at room temperature. The precipitate was immediately centrifuged at 16,000 X g for 30 min at 20°C. The supernatant fluid was decanted; the centrifuge tube was drained for about 5 min to remove as much fluid from the precipitate as possible and the precipitate was dissolved in 1 serum volume of 0.15 M NaCl containing 0.001 M NaN_3.

Buffers. 0.15 M NaCl, 0.01 M K_2HPO_4, 0.005 M KH_2PO_4, 0.001 M NaN_3 and 0.1 mg/ml, rabbit γ-globin (Schwarz/Mann, Orangeburg, NY) Hepes buffered Hanks (Flow Laboratories, Rockville, MD).

Characterization of the humoral immune response by means of fluorescence polarization. Immunoglobulin (Ig) equivalent to 10, 30 or 100 µl of serum was diluted in a fluorescence cuvette to 3 ml with buffer and the blank fluorescence measured by a fluorescence polarometer (18). Fluorescein (3, 10 or 30 µl of 10^{-6} M) was added to the diluted Ig and the solution was mixed with a Pasteur pipette. After 30 min at room temperature the fluorescence intensities and

polarizations were measured. Fluorescence parameters for free, unbounded fluorescein were measured in the presence of normal Ig only. The data were treated as described in the Appendix to give the serum antibody site concentration, the antibody-hapten binding affinity and the antibody heterogeneity index. Alternatively, the polarizations themselves can be viewed as a kind of titer assessing the immune response.

Quantification of the cellular immune response. To assess the magnitude of the cellular immune response against FO the test animals were challenged with 0.1 ml of FO (400 µg/ml in Hepes buffered Hanks) in one footpad and with the buffer alone in the contralateral footpad. Twenty-four hours later the response was quantified in two ways. In the first the immune response was graded subjectively on the basis of color and swelling on a scale of 1 through 4 and plotted as an average difference between experimental and control feet for all animals in the group (usually 5 animals). In the second method (thermometric footpad assay) thermocouples were attached with adhesive to the test and control feet and the difference in footpad temperature was measured with the circuit shown in Figure 8. In some cases the footpads were dissected after temperature measurement in order to determine the degree of correlation between temperature and histological findings.

RESULTS

The effect of pesticides on the cellular immune response was measured by two methods, the first being a visual evaluation of the intensity of inflammation and swelling of an antigen challenged footpad as compared to the contralateral pad treated with buffer alone. Figure 1 shows that by this measurement Dinoseb and

Parathion markedly depress the response while Resmethrin gives a large stimulation appearing very early after primary immunization. Methotrexate and PCNB appear also to stimulate cellular immunity but only late in the immune respone. Aroclor, piperonyl butoxide and mixed pyrethrins have little if any effect. The second method for evaluating cellular immunity involved a differential temperature measurement between the antigen-challenged and control footpads. The results of this thermometric footpad assay were found to correlate positively with visual evaluation of the degree of inflammation and with pathological findings in the antigen-challenged footpad tissue. Results of the thermometric footpad assay are shown in Fig. 2. Resmethrin as before shows an early, sometimes very large, stimulation while Methotrexate, PCNB and possibly pyrethrins give a late stimulation. Both Dinoseb and Parathion are depressive while the other pesticides show no detectable effects. The effect of size of the first dose of immunogen is obviously quite important as shown by Figs. 1 and 2. The smaller dose (0.4 mg per hamster) resulted in a much larger response than did the larger dose (4 mg).

Pesticide effects on the humoral response were assessed by fluorescence polarization measurements after adding fluorescein to an Ig preparation from serum. The polarization itself can be thought of as a titer dependent upon both antibody concentration and antibody binding affinity. The polarization titers of Fig. 3 show depression by Dinoseb and Parathion and not much effect of the other pesticides. The effect of size of immunizing dose is again evident, the smaller dose giving the larger response.

All examined preimmunization bleedings gave only background polarization vlaues (0.027, the same as in buffer alone).

As mentioned above, polarization is dependent upon both the antibody concentration and the binding affinity. These two variables together with a third variable the heterogeneity index can be segregated by an analysis of the complete titration curve. The results of these computations are shown in Figs. 4 and 5. In Fig. 4 the quantity $F_{b,max}$, which is equal to the molar concentration of antibody combining sites in a 300 fold dilution of serum, is shown for different pesticides during progression of the immune response. A marked depression can be seen for Dinoseb and Parathion with only minor differences from controls for the other pesticides. In Fig. 5 the largest effect on the binding affinity as measured by the average association constant seems to be produced by varying the size of immunizing dose. All values of K_o lay between 10^8 and 10^9 $1mole^{-1}$ which is a rather small variation.

The typical appearance of fluorescence polarization titration curves can be seen in Fig. 6 for piperonyl butoxide at 36 days. The agreement between experiment and theory is very close (chi^2 for these data is 1.19). For the other data shown in Figs. 4 and 5, the values of chi^2 varied from 0.23 to 2.2. A third variable, a, the heterogeneity index was also obtained from the titration data. The values ranged from 0.7 to 1 and are shown in Table 1 with results of all the computations.

The general physiological state of the animals was monitored by the change in body weight during the experiments (Fig. 7). Both Dinoseb and Parathion show a prolonged effect in depressing growth

while Methotrexate and Aroclor give transient depressions. The high dose of immunogen itself shows a marked depression when compared to the low dose control.

The schematic for the circuit used in the thermometric footpad assay is shown in Fig. 8.

DISCUSSION

The experimental data obtained shows that a single dose of orally administered pesticide may exert large, long-lasting effects on the immune response. The effects observed may be either stimulation or depression and may be directed selectively towards either the cellular or hurmoral immune response depending upon the pesticide.

In this study these effects were monitored by measurement of several parameters related to various aspects of the immune response to a single well-defined immunogen, fluorescein labeled ovalbumin (FO). The humoral response measured was directed against fluorescein itself while the cellular response was that directed against the entire immunogen. The effects observed as summarized in Table 2 show that the most significant findings are a marked depression of both the cellular and humoral immune response by Dinoseb and Parathion and an early and sometimes very pronounced stimulation of the cellular response by Resmethrin. The latter effect is of considerable interest in two contexts. First, Resmethrin when used with another pesticide may exaggerate any antipesticide reactions in humans or animals exposed to the two substances together. Secondly, and perhaps more important, the behavior of Resmethrin may provide an important clue towards

designing potent stimulators of the cellular immune response. Such materials could be of great value in treating bacterial, viral and certain neoplastic diseases. The action of Parathion and Dinoseb are remarkably long lasting and the depression of the immune response which they evoke could lower resistance to a variety of infectious diseases. The nature of the effect on the humoral response is seen to be chiefly on the amount of antibody produced and not upon its binding affinity. Probably the type of antibody is unchanged by these pesticides but the amount is decreased.

The results of this study provide important leads which should be followed up. First, the immunological effects of many other pesticides and organics generally should be investigated and secondly, the effects should be studied not only in the whole animal but also at the level of T-cell and B-cell activation.

TABLE 2. Summary of Effects of Pesticides on the Immune Response

Pesticide	Cellular		Humoral		
	Visual Evaluation	Thermometric Footpad	Polarization Titer	Antibody Concentration	Binding Affinity
Methotrexate	+ (late)	+ (late)	0	0	0
Aroclov	0	0	0	0	0
Dinoseb	-	-	-	-	0 to -
Parathion	-	-	-	-	0
PCNB	+ (late)	+ (late)	0	0	0
Pip. Butox.	0	0	0	0	0
Pyrethrins	0	± (late)	0	0	0 to -
Resmethrin	+ (early)	+ (early)	0	0	0 to +

(-): Depression
(+): Stimulation
(0): Little or no effect

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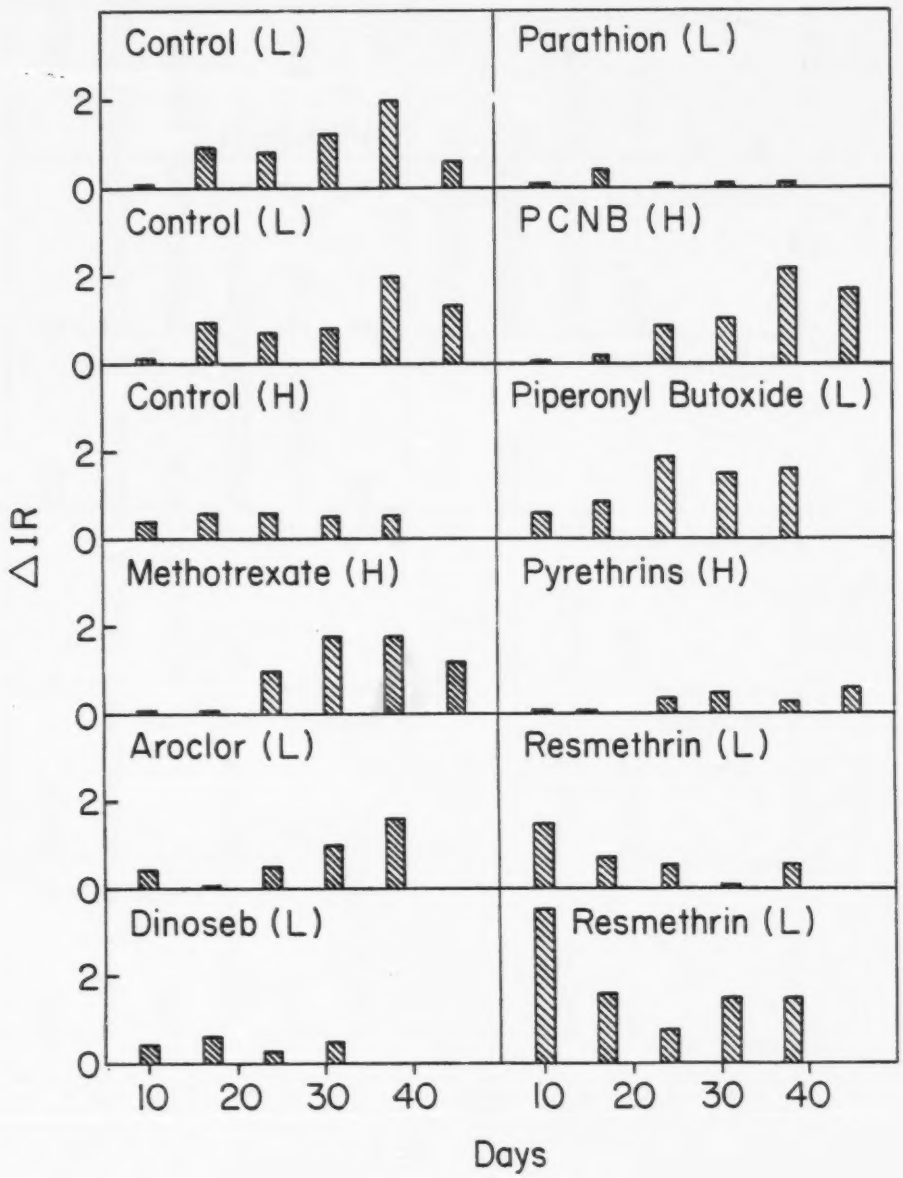

Figure 1. Effect of pesticides on the cellular immune response as measured by visual evaluation on a 1 to 4 scale of the inflammation and swelling of the footpad challenged with antigen as compared to the contralateral pad challenged with buffer alone. Animals were treated on day zero with pesticide in corn oil or with corn oil alone (control) and immunized with either a low dose (L) or a high dose (H) of FO as described in Materials and Methods. The curve of each pesticide must be compared with the appropriate control.

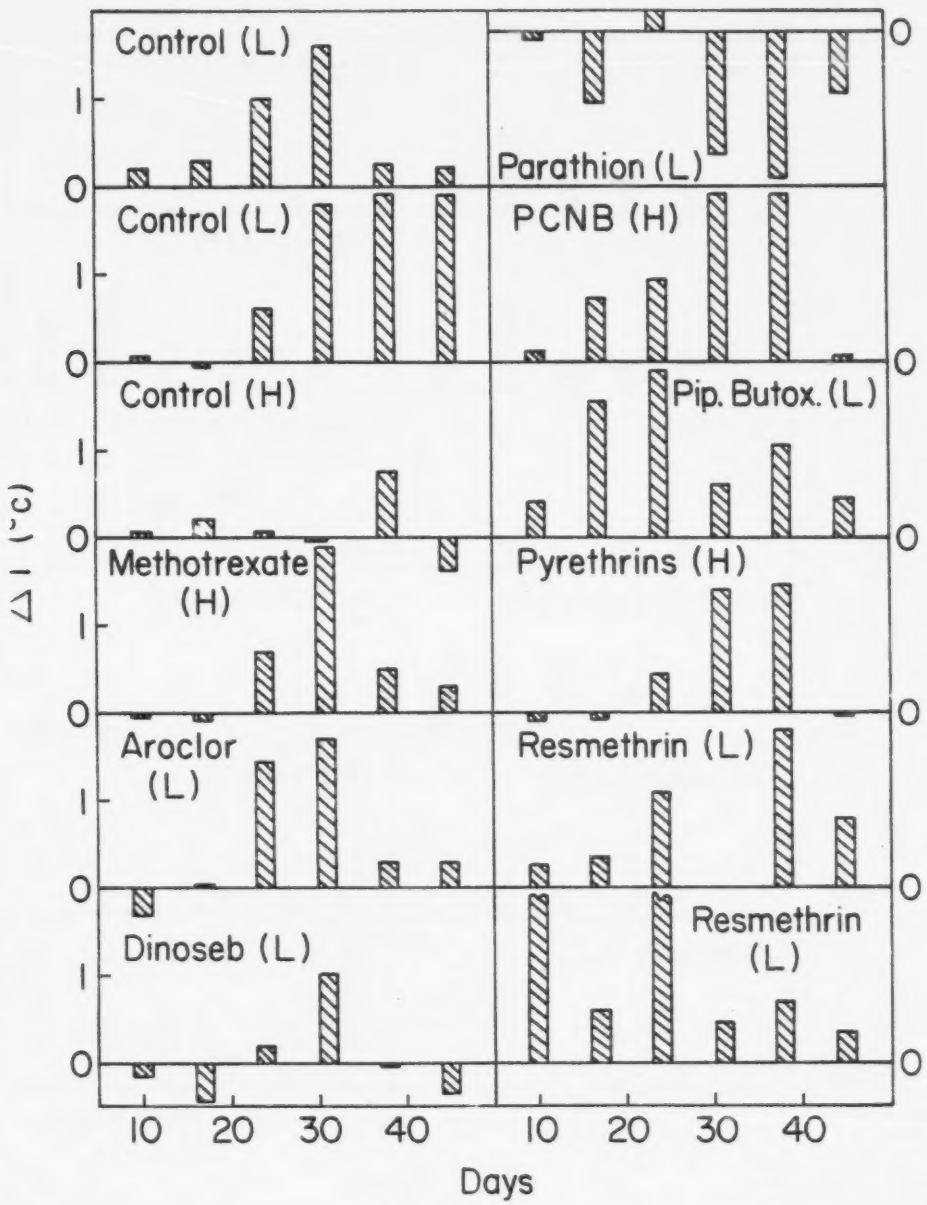

Figure 2. Effect of pesticides on the cellular immune response as measured by average values of temperature differences (ΔT) between the footpad challenged with antigen and the contralateral pad challenged with buffer alone. Animals were treated with pesticide in corn oil or with corn oil alone (control) and immunized with either a low dose (L) or a high dose (H) of FO as described under Materials and Methods. Temperature differences were measured with the circuit of Figure 8.

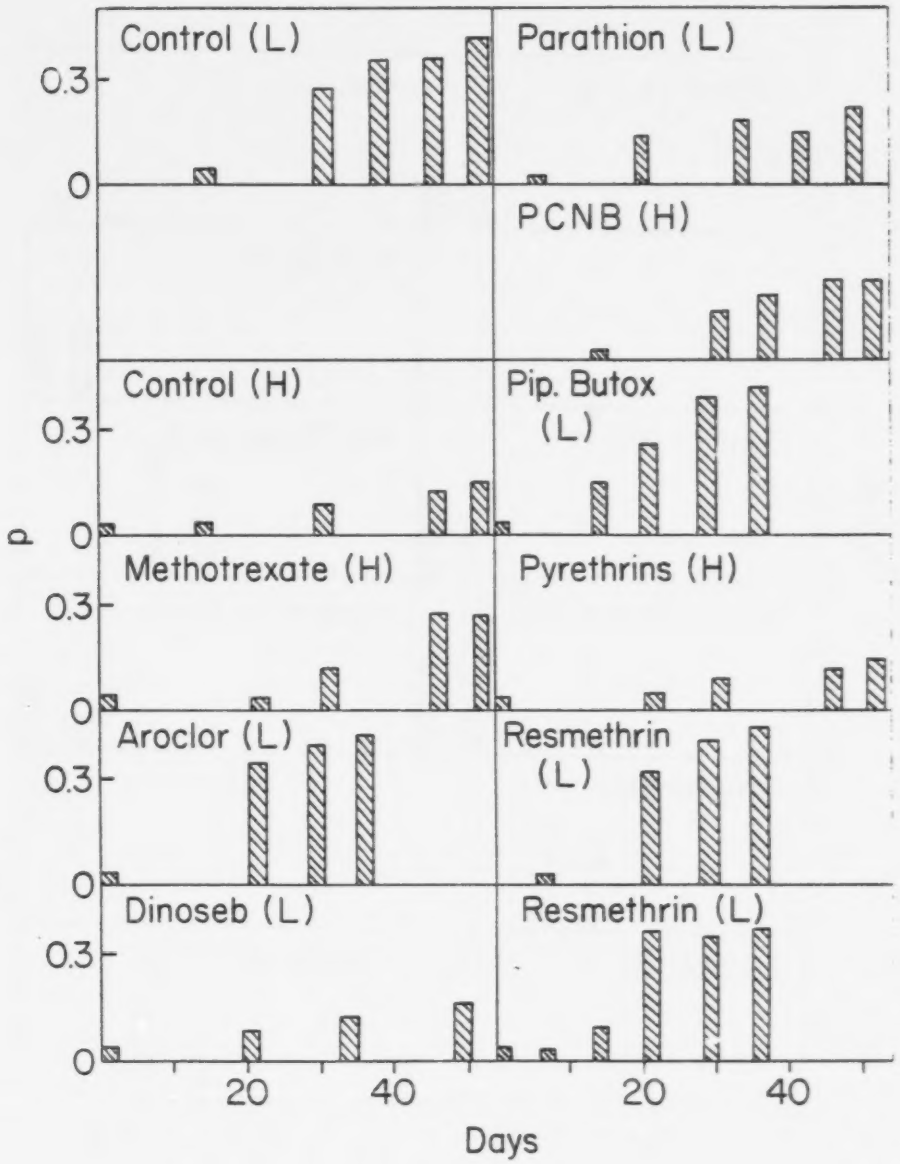

Figure 3. Effect of pesticides on the humoral immune response as measured by polarization titers. The polarization of fluorescence was measured 30 min after adding 10^{-9} M fluorescein to a solution of Ig (equivalent to 100 µl of serum) in 3 ml of buffer. The polarization is a function of both antibody concentration and antibody binding affinity and is synbatic with both of these variables. Animals were treated on day zero with pesticide in corn oil or with corn oil alone (control) and immunized with either a low dose (L) or a high dose (H) or FO as described in Materials and Methods.

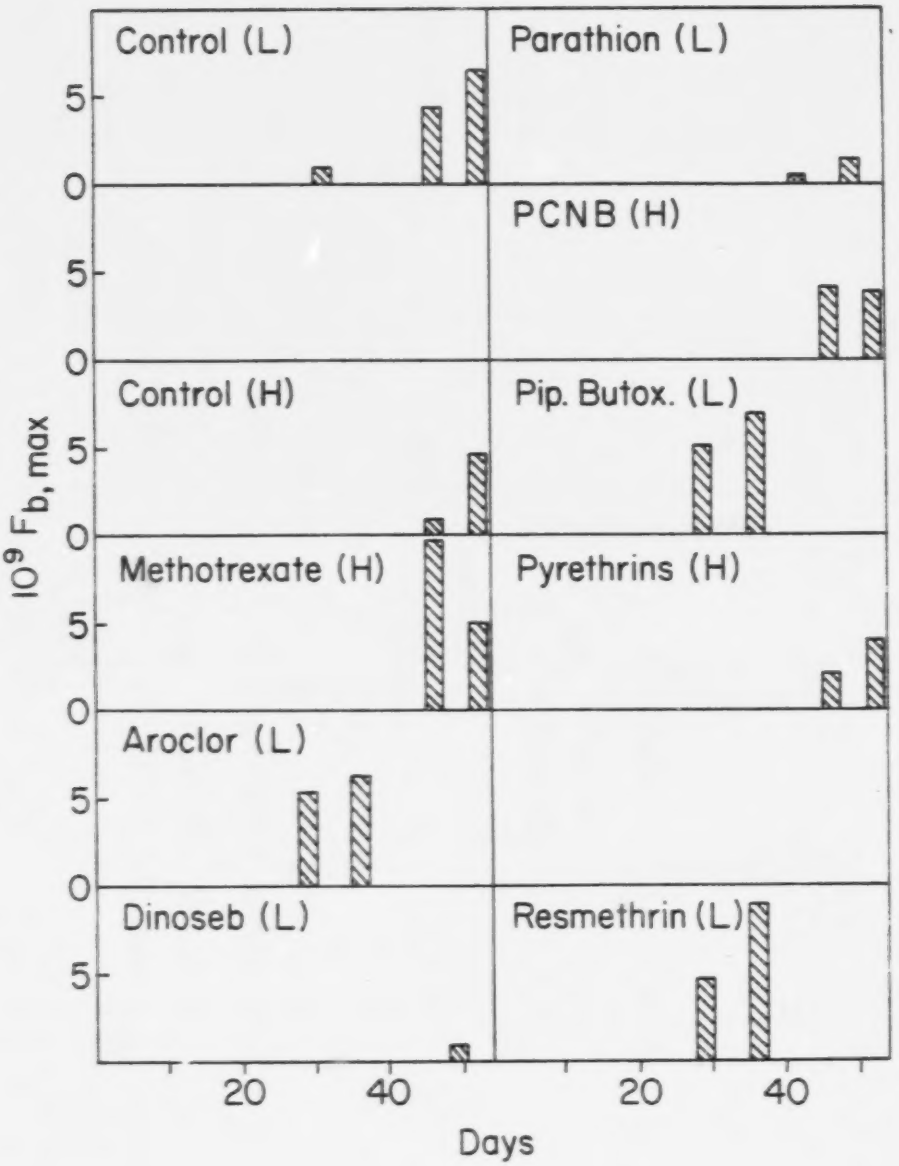

Figure 4. Effect of pesticides on the humoral immune response as measured by the concentration of serum antibody against fluorescein as a function of time after primary immunization. The quantity, $F_{b,max}$ is the molar concentration of antibody combining sites specific for fluorescein present in a 300 fold dilution of serum. Values of $F_{b,max}$ were computed from fluorescence polarization measurements, cf. Appendix.

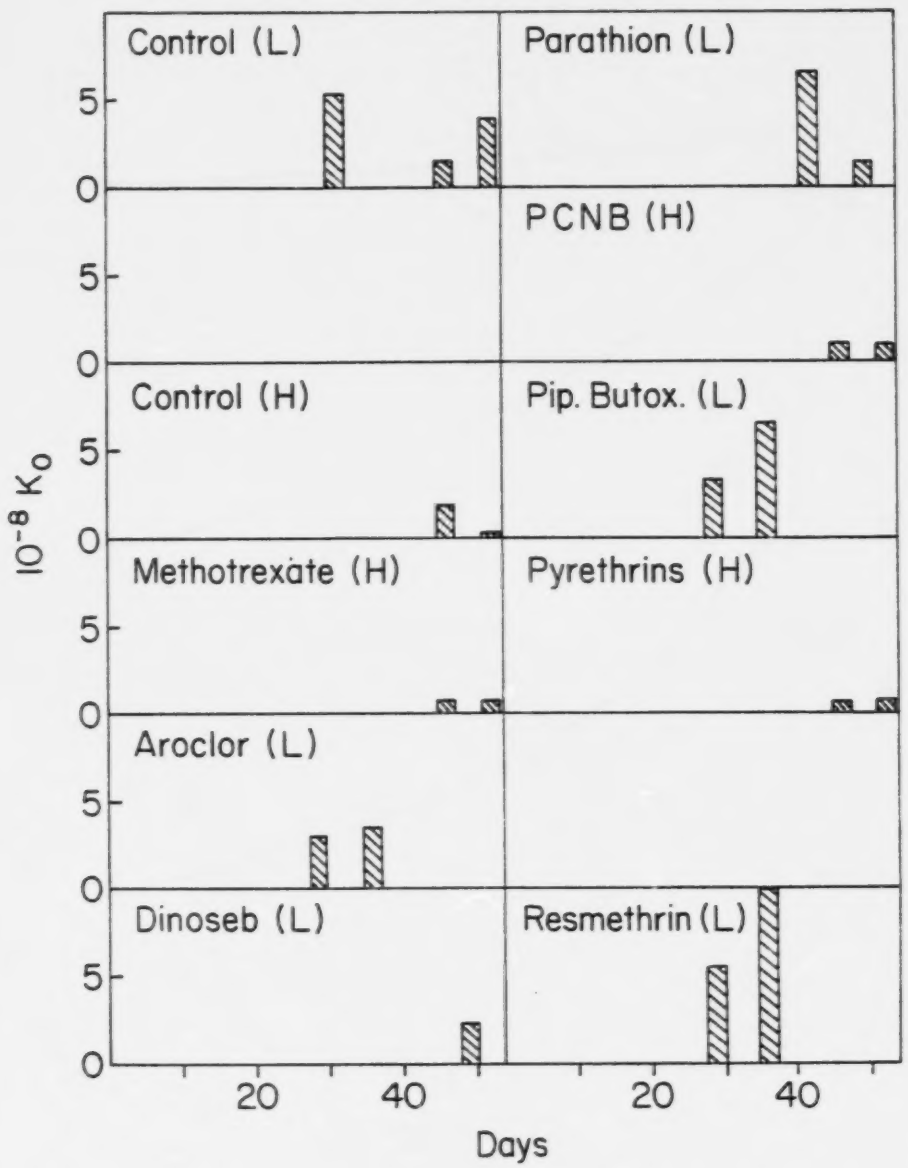

Figure 5. Effect of pesticides on the humoral immune response as measured by the average association constant (K_0) of the serum antibody present against fluorescein at different times after primary immunization. The values of K_0 were computed from fluorescence polarization measurements, cf. Appendix.

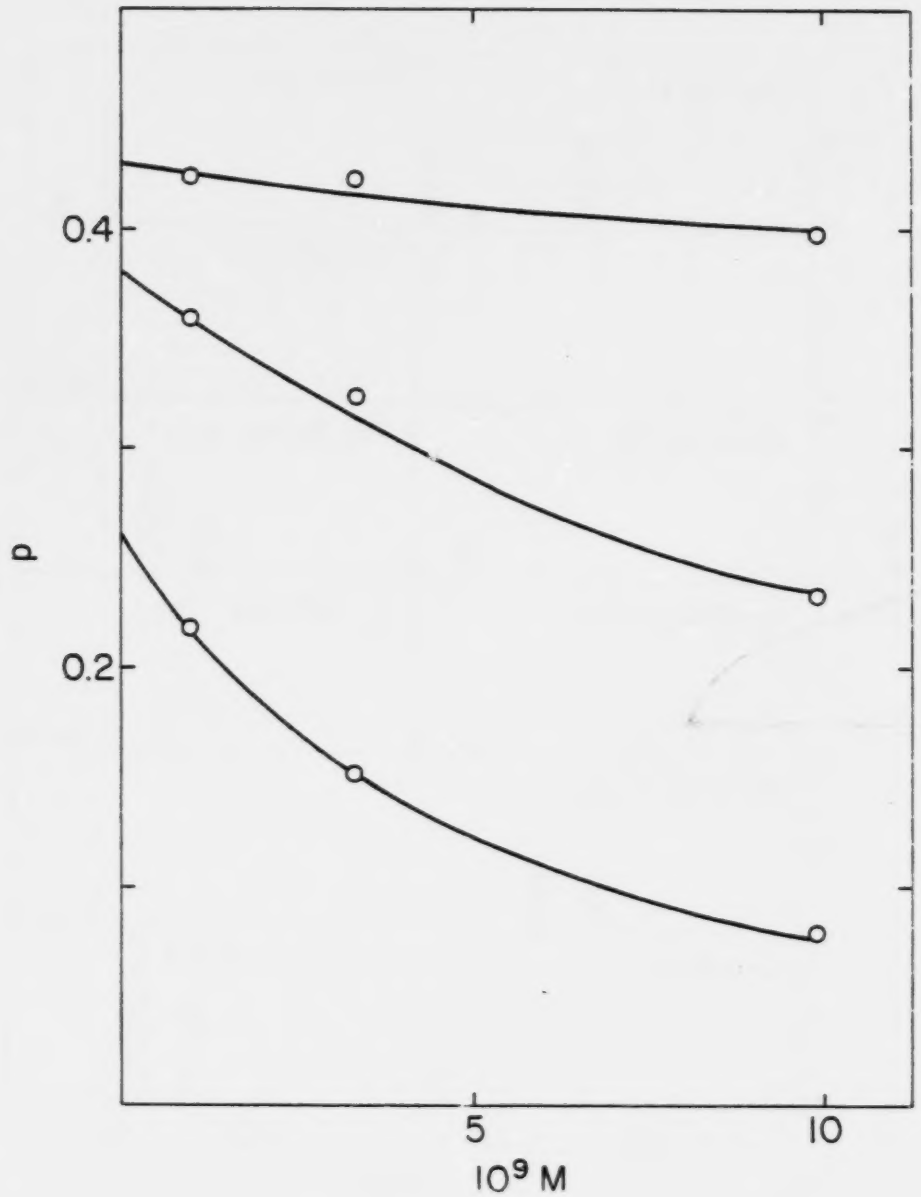

Figure 6. Typical appearance of fluorescence polarization titration curves showing polarization, p, as a function of M, the final molar concentration of fluorescein in the titration cuvette. The data points are for piperonyl butoxide at 36 days and the smooth curves are theoretical for $F_{b,max}$ = 6.74×10^{-9} M, $K_o = 5.13 \times 10^8$ M^{-1}, a = 0.77, p_f = 0.0272, p_b = 0.45 and Q_f/Q_b = 6.6. See Appendix for a discussion of these quantities.

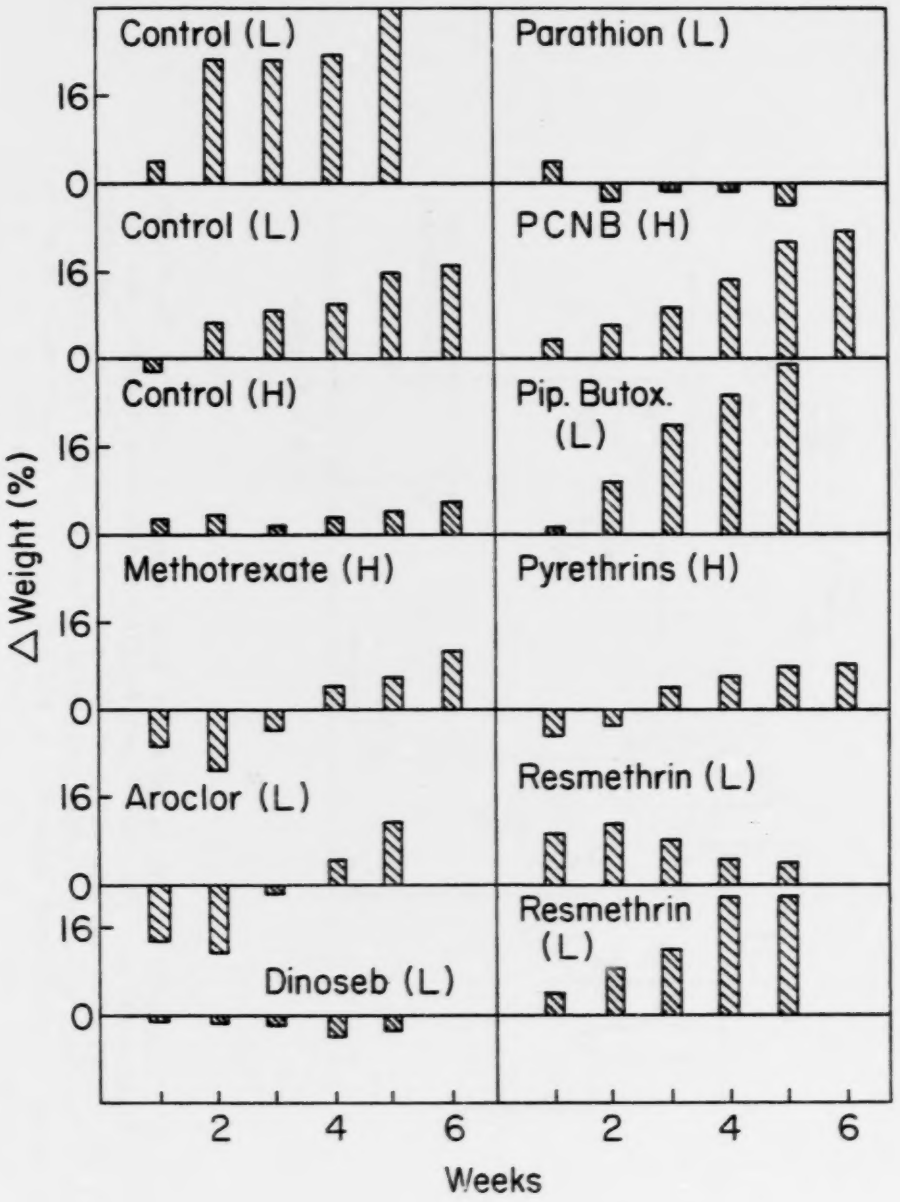

Figure 7. Effect of pesticides on body weight as a function of time after pesticide administration expressed as a percentage change of the initial weight. Animals were treated with pesticide in corn oil as indicated or with corn oil alone (control) and immunized with either a low dose (L) or a high dose (H) of F0 as described in Materials and Methods.

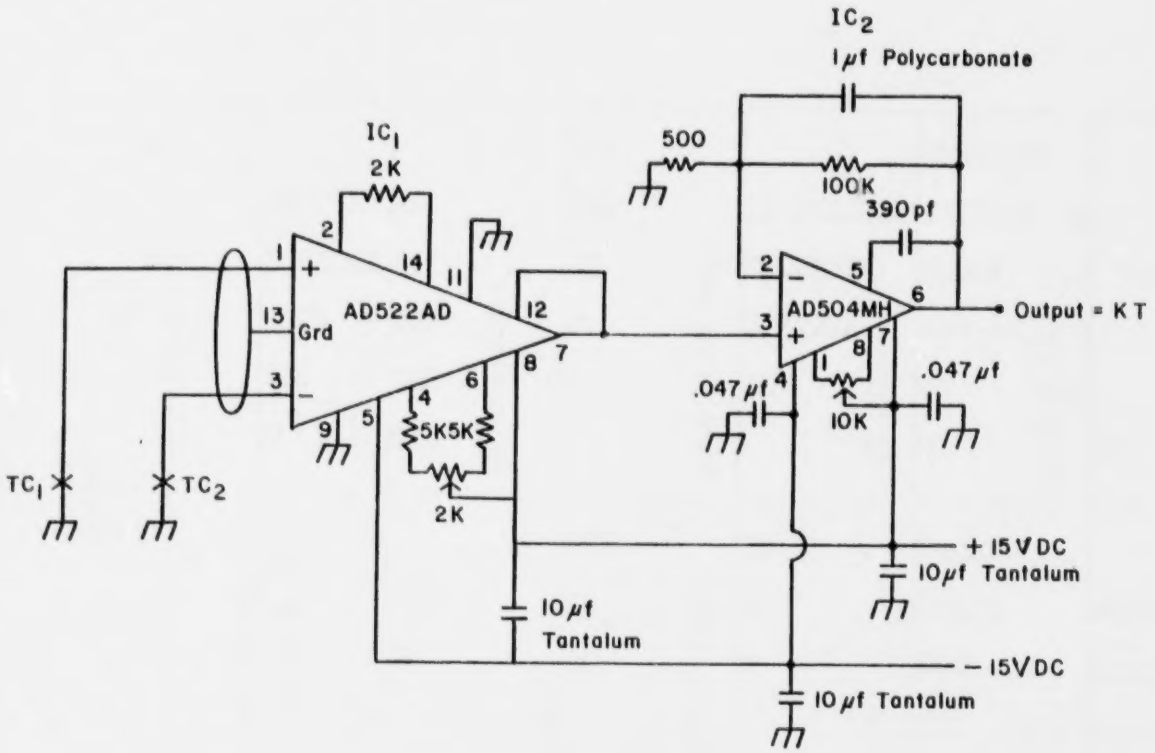

Figure 8. Schematic of the temperature measurement circuit for the thermometric footpad assay. The amplifiers AD522AD and AD504MH (Analog Devices, Norwood, Mass) give voltage gains of 100 and 201, respectively. The thermocouples Tc_1 and Tc_2 were copper constantan (Thermometrics, Northridge, CA). The output if maintained between -10 and +10 volts is proportional to ΔT and is conveniently read on a digital voltmeter. After a warmup of 15 to 30 min, the offsets were adjusted by shorting the input and adjusting the 2K potentiometer of IC_1 to give zero volts at the output. The 10K potentiometer of IC_2 does not need routine adjustment but can be set initially to give zero output after shorting the input to IC_2, i.e., the output of IC_1.

APPENDIX

In this appendix a brief review of fluorescence polarization is given together with the necessary equations for interpreting polarization data and procedures for computing several derived parameters.

The light emitted from fluorescent solutions is partially polarized; it consists of a mixture of linearly polarized and unpolarized light. The origin of this partial polarization and its implications concerning the kinetic unit carrying the fluorescent moiety can be seen from the following considerations. Classically, the emission from a single molecule may be regarded as radiation from a single oscillating dipole. This radiation has an oscillating electric field parallel to the direction of oscillation of the dipole and is said to be polarized in the same direction. If a randomly oriented assembly of molecules is excited by fully polarized light, their fluorescence is only "partially" polarized, even if the molecules are prevented from rotary Brownian motion in solution. For simplicity, assume that the direction of the absorption and emission oscillators in a single molecule are the same and that they are rigidly fixed with respect to the geometric axis of the molecule. Furthermore, assume the molecule rigidly fixed in position during the interval between absorption and emission (typically a few nanosec). The probability of absorption of light is proportional to the square of the magnitude of the component of the electric vector of the exciting light in the direction of the oscillator. If the absorption and emission oscillators are parallel the emitted light

will be partially polarized with a degree of polarization, p. This quantity is defined in terms of intensities, I, polarized either parallel of perpendicular to the incident electric field.

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \qquad (1a)$$

For randomly oriented molecules in a rigid medium, the maximum value of p observed with linearly polarized light is one-half. If, instead of being rigidly fixed, the molecules are subject to rotary Brownian motion, the molecular rotation taking place between the times of absorption and emission may be expected to result in values of p lying between one-half and zero. The extent of this rotation is a function of molecular dimensions and structure, solvent and temperature. Low molecular weight compounds such as fluorescein will give rise to virtually completely depolarized fluorescence; some polarization will be retained as molecular size increases. Considering two molecules of equal size, the fluorescence of the more asymmetric rigid structure will be more highly polarized.

The essential feature of applying these phenomena to antibody hapten binding consists in observing the degree of polarization and intensity of the fluorescent light when measured quantities of the hapten and antibody are allowed to interact. Reaction results in an increase in size of the fluorescent kinetic unit and in a retardation of the rotary Brownian motion manifested as an increase in the polarization of fluorescence. From measurements of polarization the final, derived parameters which can be obtained are 1) binding site concentration, 2) an average

association constant and 3) an index of the heterogeneity of the binding sites. The general type of reaction assumed is that in which a fluorescent ligand $\mathcal{F}$ binds to a receptor $\mathcal{R}$, to reversibly form a complex $\mathcal{F}\mathcal{R}$:

$$\mathcal{F} + \mathcal{R} \rightleftharpoons \mathcal{F}\mathcal{R} \qquad (2a)$$

Symbols

a, heterogeneity index

b, subscript indicating "bound"

F, molar concentration of $\mathcal{F}$

f, subscript indicating "free"

$F_{b,max}$ The maximum value of F_b; taken to be equal to the total molar concentration of receptor sites

M, the total molar concentration of $\mathcal{F}$ in both free and bound forms (AM in computer program)

p, the polarization of the excess fluorescence, i.e., $p = (\Delta v - \Delta h)/(\Delta v + \Delta h)$, where Δv and Δh are the intensities in arbitrary units of the components in the excess fluorescence (above that of the blank) polarized in the vertical and horizontal directions, respectively

Q, molar fluorescence of a mixture of free and bound forms of $\mathcal{F}$ as they exist in a solution under observation, i.e., $Q = (\Delta v + \Delta h)/M$

Fluorescence polarization and intensity measurements provide a direct and rapid measure of the bound/free ratio:

$$\frac{F_b}{F_f} = \frac{Q_f}{Q_b}\left(\frac{p - p_f}{p_b - p}\right) \qquad (3a)$$

and

$$\frac{F_b}{F_f} = \frac{Q_f - Q}{Q - Q_b} \qquad (4a)$$

In order to utilize these equations, the constants Q_f, Q_b, p_f and p_b must be determined for a particular system under study. No problem is posed in finding Q_f and p_f, since these come directly from a measurement on the labeled component alone. The determination of Q_b and p_b, however, implies measurements on a state in which the fluorescent labeled material is completely bound to its complementary partner. Since complete binding cannot be realized physically, an extrapolation is involved. If equilibrium values of p plotted against M are extrapolated to M = O, p approaches a limit, p´. Values of p´ for different antibody concentrations plotted against (p´ - p_f) divided by antibody concentration in any arbitrary convenient units give p_b as the intercept of a straight line, for classical mass law (18).

$$p' = p_b - \frac{Q_f(p' - p_f)}{Q_b KF_{b,max}} \qquad (5a)$$

This procedure makes it unnecessary to know absolute values of $KF_{b,max}$ beforehand. A similar relationship facilitates the determination of Q_b:

$$Q' = Q_b + \frac{Q_f - Q'}{KF_{b,max}} \qquad (6a)$$

In the program given below the measured values of M, P. Q, P_f, Q_f and relative antibody concentrations (AB) with or without tentative estimates of P_b and Q_b and used in an iterative computation to derive the final best values of P_b, Q_b, K_o, a

and $F_{b,max}$. These computations are based upon achieving a chi-square fit of the data to the Sips equation (19,20) which defines F_f as:

$$F_f = \frac{1}{K_o} \left[\frac{F_b}{F_{b,max} - F_b} \right]^{1/a} \qquad (7a)$$

Substituting $M = F_b + F_f$ into eq. (3a) and rearranging gives:

$$p = \frac{(P_f Q_f - P_b Q_b) F_f + P_b Q_b M}{(Q_f - Q_b) F_f + Q_b M} \qquad (8a)$$

Inspection of eq. (7a) and (8a) shows that there are five unknowns, $F_{b,max}$, a, K_o, P_b and Q_b to be evaluated from measured individual values of P, M and Q, viz. P_i, M_i and Q_i and from the measured values of P_f and Q_f.

In the procedure given below the user may either specify initial estimates for these unknowns along with measured molarity and polarization data, or the user can allow the program POLAR to make the initial estimates. Once initial estimates have been made for the five parameters, the program proceeds to improve these estimates in an iterative fashion until a stopping criterion specified by the user is met. The measure of goodness to fit to eq. (8a) is a modified chi-square defined by:

$$\text{CHISQ} = \text{sum} \frac{\left(P_i - P_{calc,i}\right)^2}{0.01} \qquad (9a)$$

where P is the measured polarization and P_{calc} is the value computed from eq. (8a) given the current estimates for the five parameters. The iterative improvement performed by POLAR consists of repeatedly moving away from the current best estimate of each of the parameters either by a user specified value (if

Q = molar fluorescence

Using these data, the program proceeds to:

1. Extrapolate each set of P and Q data to a zero molarity value (called P' and Q') using the subroutine FLAGR which performs Lagrange interpolation. Therefore, for each antibody, there is P' = P(0) and Q' = Q(0) which are put in the arrays APE and QPE.

2. Obtain a least squares fit to a straight line by SQRL which assembles and solves the normal equations to obtain initial estimates of P_b and Q_b:

$$P' = \text{slope} \left[\frac{(P' - P_f)}{AB}\right] + P_b \quad \text{and}$$

$$Q' = \text{slope} \left[\frac{(Q_f - Q')}{AB}\right] + Q_b.$$

3. Iteratively find initial estimates for $F_{b,max}$, K_o, and a (renamed in the program: FBMAX, OK,A).

4. Use the chi-square fit to improve these initial estimates.

Option 2. The program reads in P_f, Q_f, N (= number of antibody concentrations) and RANGE.

Then for each antibody the following data are read:

AB = antibody (scaled)

NP = number of molarities at which P has been measured

followed by NP sets of:

AM ≡ M = molarity

P = polarization

the user provided the initial estimates) or by 10% of the current estimate (if POLAR computed the initial estimates), computing the chi-square value that results and keeping track of the parameters that give the smallest chi-square value. As the iteration proceeds, the amount that is being added or subtracted from the current best estimate is halved as the value of the parameter nears an optimal value. The iteration continues until all the parameters satisfy the following test, where RANGE is a value entered by the user at the onset:

$$\frac{\text{increment}}{\text{current value}} \longleftarrow \text{RANGE} \qquad \text{for all five parameters} \tag{10a}$$

A frequently occurring computation in this search for optimal parameters is the value of F_{fi}. This is accomplished by a root-finding routine ZEROIN which will find the zero of a function, given an initial interval in which that root must lie. Equation (7a) for F_{fi} is written as follows to avoid the singularity present when F_{bi} approaches $F_{b,max}$.

$$(K_o F_{fi})^a \qquad (F_{b,max} - AM + F_{fi}) - AM + F_{fi} = 0 \tag{11a}$$

using the fact that molarity, $AM \equiv M = F_{bi} + F_{fi}$.

The program POLAR operates in two modes depending on the amount of data the user can supply.

Option 1. The program reads in P_f, Q_f, N (= number of antibody concentrations) and RANGE. Then, for each antibody concentration the following data are read:

AB = antibody concentration (scaled so smallest is 1)

NP = number of molarities at which p and Q have been measured, followed by NP sets of:

AM ≡ M = molarity

P = polarization

The program then reads in initial estimates provided by the user for the five parameters,

FBMAX, QF/QB (only ratio is important), A, OK, PB,

followed by five values specifying how much the current values of the parameters are to be varied in optimizing the chi square fit. If the parameter is not to be changed by the program, then a zero should be entered for the corresponding variance. Otherwise a good value to use is 10% of the original estimate. The program proceeds then to improve these initial estimates using the chi-square criterion. As an aid to the user, a print out of the entire program with subroutines as well as of the runs for piperonyl butoxide at 36 days using first option 1 and then option 2 are given below.

```
      PROGRAM POLAR (INPUT,LIST,TESTER,TAPE1=TESTER,TAPE3=LIST)
C
CC  N=NUMBER OF SETS OF DATA POINTS
CC
      DIMENSION QPE(15),QPA(15),NP(15),P(30,15),AM(30,15),
     1 APE(15),APA(15),AB(15),Q(30,15),FF(30,15),FB(30,15),X(15),
     2 OKS(450),P1(6),PVAR(6),FIX(6),PT(6,6),PC(30,15),PFIT(6),TITL(4)
      EXTERNAL F
      COMMON A,AMOL,FBM,OK
C
CC  READ HEADING AND INPUT.  HEADING IS 40 SPACES.
C
      REWIND 1
   99 READ (1,59)TITL,INDIC
   59 FORMAT(4A10,I2)
      IF(INDIC)800,64,314
   64 WRITE(3,68)
   68 FORMAT(1H1)
      WRITE(3,59)TITL
C
CC  RANGE IS A QUIT CRITERION.  IF ALL PARAMETERS ARE +/- RANGE, THEN END.
C
      READ(1,100)N,QF,PF,RANGE
      WRITE (3,3) N,PF,QF
    3 FORMAT(/,5H WITH,I3,19H ANTIBODIES   PF = ,F10.4,5X,4HQF= ,F10.4)
  100 FORMAT(I2,3F10.5)
C
CC  NP=NUMBER OF POINTS IN EACH SET, IE. MUST BE ONE VALUE OF NP FOR EACH SET
CC  ONE ,,NP,, AND ONE AB VALUE PRECEED EACH SET OF POINTS
C
      DO 4 KK=1,N
      READ(1,101)NP(KK),AB(KK)
  101 FORMAT(I2,F10.5)
C
CC  ONE VALUE OF MOLARITY AND THE CORRESPONDING P AND Q VALUES ARE TO BE PUNCHED
CC  ON EACH CARD.  AM=MOLARITY,P=POLARIZATION, AND Q=QUENCHING.
C
      NB=NP(KK)
    4 READ (1,102) (AM(I,KK),P(I,KK),Q(I,KK),I=1,NB)
  102 FORMAT(3F10.5)
      DO 7 KK=1,N
      NB=NP(KK)
C
CC  USE THE FUNCTION FLAGR TO EXTRAPOLATE P'S TO ZERO
C
      APE(KK)=FLAGR(AM(1,KK),P(1,KK),0.,NB-1,NB)
C
CC  USE THE FUNCTION FLAGR TO EXTRAPOLATE  Q'S TO ZERO
C
      QPE(KK)=FLAGR(AM(1,KK),Q(1,KK),0.,NB-1,NB)
      WRITE (3,63)
   63 FORMAT(1HO)
      WRITE (3,104) KK,KK
  104 FORMAT(//34H THE FIRST TERM IS P' FOR DATA SET  ,I2,2X,
     134H     FIRST TERM IS Q' FOR DATA SET  ,I2)
    7 WRITE (3,103)  APE(KK),QPE(KK),(P(I,KK),Q(I,KK),I=1,NB)
  103 FORMAT(3X, F10.6,32X,3X,F10.5)
```

```
C
C   FORM INDEPENDENT VARIABLES, APA AND QPA, FOR LEAST SQUARES FIT
C
      DO 21 I=1,N
         APA(I) = (APE(I) - PF)/AB(I)
         QPA(I) = (QF-QPE(I))/AB(I)
21       CONTINUE
C
C   LEAST SQUARES FIT FOR PB AND QB
C
      CALL SORL (APA,APE,N,SLOPE,PB,STDERR)
      CALL SORL (QPA,QPE,N,SLOQ,QB,STDQRR)
C
      DO 22 IMQ=1,N
      IR=NP(IMQ)
      DO 22 IZZZ=1,IR
      PBIG=P(IZZZ,IMQ)
      IF(PB-PBIG)20,20,22
20    PB=1.2*PBIG
22    CONTINUE
C
C     PRINT THE OUTPUT
C
      WRITE (3,107) PB,QB,SLOPE,SLOQ
107   FORMAT(//6H PB = ,F8.5,34X,6H QB = ,F8.4,/,8H SLOPE= ,E15.5,26X,
     *  7HSLOPE= ,F8.5)
      WRITE (3,108)  STDERR,STDQRR
108   FORMAT (18H STANDARD ERROR =  , F7.5,23X,18H STANDARD ERROR = ,
     1F7.5)
C
C   CALCULATE FF AND FB.
C
      DO 121 J=1,N
      NB=NP(J)
      DO 121 I=1,NB
      FF(I,J)=QB*AM(I,J)*(PB-P(I,J))/(P(1,J)*(QF-QB)-PF*QF+PB*QB)
121   FB(I,J)=AM(I,J)-FF(I,J)
      GOTO 817
      WRITE (3,812)
812   FORMAT(//,70H1CALCULATED VALUES OF FF AND FB, BASED ON FIRST ESTIM
     1ATES OF PB AND QB.//,71H                ANTIBODY                MOLARITY
     2          FF                FB              )
      DO 814 JO=1,N
      NPOL=NP(JO)
      WRITE (3,813) AB(JO)
813   FORMAT(3H     ,E14.8)
      WRITE (3,815) (AM(I,JO),FF(I,JO),FB(I,JO),I=1,NPOL)
815   FORMAT(23X,E14.8,6X,E12.4,6X,E12.4)
      WRITE (3,816)
816   FORMAT(1HO)
814   CONTINUE
C
C   DETERMINE APPROXIMATE VALUES FOR A, KD, AND FBMAX.....
C
817   SIGMA = 1000.
      A=.9
      SIZE=.1
```

```
      123 NK1=0
          DO 122 J=1,N
          NB=NP(J)-1
          DO 122 I=1,NB
          NK1=NK1+1
          TOK=((FB(I,J)/FF(I,J)**A-FB(I+1,J)/FF(I+1,J)**A)/(FB(I+1,J)-FB(I,J
         1)))
          IF(TOK)124,124,125
      124 OKS(NK1)=0.0
          GO TO 122
      125 OKS(NK1)=TOK**(1./A)
      122 CONTINUE
          ENK=NK1-1
          NZIP=NK1-1
          SUM = 0.0
          DO 126 I=1,NZIP
          RASMA = OKS(I) + OKS(I+1)
          IF(RASMA) 822,822,821
      821 ROS=SQRT(4.*((OKS(I  )-OKS(I+1  ))/(OKS(I  )+OKS(I+1  )))**2)
          SUM = SUM + ROS
          GO TO 126
      822 SUM = SUM + 2.0
      126 CONTINUE
          SIG=SUM/ENK
          TEMPK=0.0
          O=0.0
          DO 127 I=1,NK1
          IF(OKS(I)) 127,127,128
      128 TEMPK=TEMPK+OKS(I)
          O=O+1.0
      127 CONTINUE
          IF(SIGMA-SIG)130,130,129
      129 OK=TEMPK/O
          SIGMA=SIG
          AVAL=A
          A=A-SIZE
          GO TO 123
      130 IF(SIZE-.01)132,132,131
      131 SIZE=.01
          A=A+.09
          GO TO 123
C
C  SOLVE FOR FBMAX.
C
      132 FBMAX=0.0
          DENOM=0.0
          DO 133 J=1,N
          NB=NP(J)
          DO 133 I=1,NB
          FBMAX=FBMAX+FB(I,J)*(I.+1./(OK*FF(I,J)))**AVAL)
      133 DENOM=DENOM+1.0
          FBMAX=FBMAX/DENOM
          A=AVAL
          WRITE (3,811) A,OK,FBMAX
      811 FORMAT(21H-FIRST  APPROXIMATIONS  ,/,12H          A = ,F10.6,/,
         112H         KO = ,E10.4,/,12H     FBMAX = ,E10.4)
C
```

```
C  BEGIN FITTING CONSTANTS FOR BEST CALCULATED P VALUES IN THE LEAST SQUARES
C  SENSE.
C
      P1(1)=FBMAX
      P1(2)=QB
      P1(3)=A
      P1(4)=OK
      P1(5)=PB
C
C  PVAR(I)=+/- MAXIMUM ALLOWABLE EXCURSION FOR THE I'TH FIRST GUESS.
C
  602 PVAR(2)=.1*P1(2)
  605 PVAR(5)=.1*P1(5)
  607 PVAR(1)=.1*P1(1)
      PVAR(3)=.075*P1(3)
      PVAR(4)=.1*P1(4)
      WRITE (3,88)
      GOTO 608
  800 CONTINUE
C
C  THIS IS THE BEGINNING OF OPTION TWO.  READ IN THE
C  APPROXIMATIONS FOR FBMAX, QF/QB, A, KO, AND PB
C  FOLLOWED BY THE AMOUNT YOU WANT THE CHI-SQUARE FIT
C  ROUTINE TO USE TO VARY THE VALUE.
C
C  NOTE - ENTERING AT ZERO FOR THIS VARIANCE WILL FORCE THE PROGRAM
C  TO USE THE ESTIMATE THE USER HAS PROVIDED, AND NOT CHANGE IT.
C
      WRITE (3,88)
      WRITE (3,59) TITL,INDIC
C
      READ (1,100) N,QF,PF,RANGE
      WRITE (3,3) N,QF,PF
      DO 805 KK=1,N
         READ (1,101) NP(KK),AB(KK)
         NB=NP(KK)
  805    READ (1,807) (AM(I,KK),P(I,KK),I=1,NB)
  807    FORMAT (2F10.5)
      READ (1,810) (P1(I),I=1,5)
  810 FORMAT (5E10.3)
      READ (1,810) (PVAR(I),I=1,5)
      P1(2) = QF/P1(2)
      P1(6)=PF
      PVAR(6)=0.
C
C  BEGINNING OF THE ITERATIVE CHI-SQUARE IMPROVEMENT ROUTINE
C
  608 CHISQ = 1000.
  606 DO 620 I=1,5
      FIX(I)=PVAR(I)/P1(I)
      PT(I,1)=P1(I)-PVAR(I)
      PT(I,2) = P1(I) + PVAR(I)
  620 CONTINUE
C
C  COMPUTE ALL POSSIBLE COMBINATIONS AND CHI-SQUARE VALUES)
C
      IF (PT(3,2) .GT. 1.) PT(3,2)=1.
```

```
      DO 299 J1 = 1,2
      FBMAX = PT(1,J1)
      DO 299 K1 = 1,2
      QB = PT(2,K1)
      DO 299 L1 = 1,2
      A = PT(3,L1)
      DO 299 M1 = 1,2
      QK = PT(4,M1)
      DO 299 N1 = 1,2
      PB = PT(5,N1)
      SUMSQ = 0.0
      R = 0.0
      NFLAGS = 0
      DO 290 NN = 1,N
      FBM = FBMAX*AB(NN)
      NQ = NP(NN)
      DO 290 MM = 1,NQ
      AMOL = AM(MM,NN)
      B=1.E-12
      C=1.E-7
      FTMV = ZERO(N (B,C,F,1.E-12)
      IF (FTMV .EQ. 1.E-12) GOTO 290
  260 PC(MM,NN)=((PF*QF-PB*QB)*FTMV+PB*QB*AMOL)/((QF-QB)*FTMV+QB*AMOL)
      R = R+1.
      SUMSQ = SUMSQ + ( P(MM,NN) - PC(MM,NN))**2
  290 CONTINUE
      CHSQ = 100. * SQRT (SUMSQ)
      IF (CHSQ .EQ. 0.) GOTO 299
      IF(CHISQ-CHSQ) 299,299,291
  291 CHISQ = CHSQ
      KUTBAK = 1
      PFIT(1) = FBMAX
      PFIT(2) = QB
      PFIT(3) = A
      PFIT(4) = QK
      PFIT(5) = PB
      PFIT(6)=PF
  299 CONTINUE
      NZ = 5
  409 IF(NZ-0) 410,420,410
  410 IF(FIX(NZ)-RANGE) 411,420,420
  411 NZ = NZ - 1
      IF(NZ - 0) 409,500,409
  420 CONTINUE
      DO 424 M = 1,5
  424 P1(M) = PFIT(M)
      IF(KUTBAK - 1) 421,423,423
  421 KUTBAK = 0
      DO 422 M = 1,5
  422 PVAR(M) = .5*PVAR(M)
  423 KUTBAK = 0
      GO TO 606
C
C  PRINT FINAL OUTPUTS.
C
  500 CONTINUE
      PFIT(2)=QF/QB
```

```
      PVAR(2)*QF/(QB-PVAR(2))-QF/QB
      WRITE (3,799)
  799 FORMAT(48H                                        LEAST SQUARES FIT        )
      WRITE (3,305)  CHISQ,(PFIT(I),PVAR(I),I = 1,6)
  305 FORMAT(15H0 XI-SQUARED = ,E14.8,///,
     112H     FBMAX = ,E10.4,7H  +/-  ,E10.4,/,
     212H     QF/QB = ,F10.4,7H  +/-  ,E10.4,/,
     312H         A = ,F10.6,7H  +/-  ,E10.4,/,
     412H        KD = ,E10.4,7H  +/-  ,E10.4,/,
     512H        PB = ,F10.6,7H  +/-  ,E10.4,/,
     612H        PF = ,F10.6,7H  +/-  ,E10.4)
C
CCC  COMPUTE AND PRINT THE BEST POLARIZATION VALUES.
C
      QB=QF/PFIT(2)
      A = PFIT(3)
      DK = PFIT(4)
      PB = PFIT(5)
      PF=PFIT(6)
      DO 790 NN = 1,N
      FBM = PFIT(1)*AB(NN)
      NQ = NP(NN)
      DO 790 MM = 1,NQ
      AMOL = AM(MM,NN)
      B = 1.E-12
      C = 1.E-7
      FTMV = ZEROIN (B,C,F,1.E-12)
      IF (FTMV .NE. 1.E-12) GOTO 760
      PC (MM,NN) = 0.
      GOTO 790
  760 PC(MM,NN)=((PF*QF-PB*QB)*FTMV+PB*QB*AMOL)/((QF-QB)*FTMV+QB*AMOL)
  790 CONTINUE
      WRITE (3,306)
  306 FORMAT(1H0)
      WRITE (3,307)
  307 FORMAT(74H          ANTIBODY              MOLARITY              P OBSERVED
     1       P CALCULATED                                )
      DO 310  JO = 1,N
      NPOL = NP(JO)
      WRITE (3,308) AB(JO)
  308 FORMAT(3H     ,E14.8)
      WRITE (3,309)(AM(IZID,JO),  P(IZID,JO),PC(IZID,JO),IZID=1,NPOL)
  309 FORMAT(    23X,E14.8,5X,F10.6,10X,F10.6 )
      WRITE (3,311)
  311 FORMAT(1H0)
  310 CONTINUE
      GO TO 99
  314 STOP
      END
```

```
C
      SUBROUTINE SQRL(X,Y,N,A,B,STDERR)
C
C  COMPUTE LEAST SQUARES FIT TO LINEAR EQUATION
C
C                Y = A X + B
C
C  USING NORMAL EQUATIONS (ONLY BECAUSE SIMPLE LINEAR MODEL USED)
C
C        N       SUMX       B              SUMY
C
C     SUMX      SUMXX       A              SUMXY
C
      DIMENSION X(30),Y(30),CALCY(30),RESID(30)
C
CC    COMPUTE SUMS
C
      SUMX=0.0
      SUMY=0.0
      SUMXX=0.0
      SUMXY=0.0
      DO 22 I=1,N
      SUMX=SUMX+X(I)
      SUMY=SUMY+Y(I)
      SUMXX=SUMXX+X(I)*X(I)
   22 SUMXY=SUMXY+X(I)*Y(I)
C
CC    COMPUTE PARAMETERS A AND B
C
      ORD=N
      DENOM=ORD*SUMXX-SUMX**2
      IF(DENOM) 24,25,24
   24 A=(ORD*SUMXY-SUMX*SUMY)/DENOM
      B=(SUMY*SUMXX-SUMX*SUMXY)/DENOM
C
CC    COMPUTE RESIDUES
C
      SUMRES=0.0
      DO 23 I=1,N
      CALCY(I)=A*X(I)+B
      RESID(I)=Y(I)-CALCY(I)
   23 SUMRES=SUMRES+RESID(I)**2
C
CC    COMPUTE THE ERRORS
C
      STDERR=SQRT(SUMRES/(ORD-2.))
      GO TO 26
   25 B=SUMY/ORD
   26 IF(B) 27,27,28
   27 B=1.
   28 RETURN
      END
```

```
CXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
      REAL FUNCTION ZERODN (AX,BX,F,TOL)
C
C  ROOT FINDER FROM    COMPUTER METHODS FOR MATHEMATICAL COMPUTATIONS
C                BY    FORSYTHE, MALCOLM AND MOLER
C                      PRENTICE-HALL, 1977
C
C  AX AND BX SHOULD BRACKET THE REGION IN WHICH THE ROOT IS TO BE
C  FOUND.  F IS THE NAME OF AN EXTERNAL FUNCTION PROVIDED BY USER
C  WHICH SPECIFIES THE FUNCTION WHOSE ROOT IS SOUGHT.  TOL IS A
C  USER DEFINED ACCURACY REQUEST.
C
C  NOTE:  MACHINE DEPENDENT ROUNDOFF ERROR EPS
C
      EPS = 7.E-14
C  INITIALIZATION
      A=AX
      B=BX
      FA=F(A)
      FB=F(B)
C  BEGIN STEP
   20 C=A
      FC=FA
      D=B-A
      E=D
   30 IF (ABS(FC) .GE. ABS(FB)) GOTO 40
      A=B
      B=C
      C=A
      FA=FB
      FB=FC
      FC=FA
C  CONVERGENCE TEST
   40 TOL1=2.*EPS*ABS(B) + .5*TOL
      XM=.5*(C-B)
      IF (ABS(XM) .LE. TOL1) GOTO 90
      IF (FB .EQ. 0.) GOTO 90
C  IS BISECTION NECESSARY
      IF (ABS(E) .LT. TOL1) GOTO 70
      IF (ABS(FA) .LE. ABS(FB)) GOTO 70
C  IS QUADRATIC INTERPOLATION POSSIBLE
      IF (A .NE. C) GOTO 50
C  LINEAR INTERPOLATION
      S=FB/FA
      P=2.*XM*S
      Q=1.-S
      GOTO 60
C  INVERSE QUADRATIC INTERPOLATION
   50 Q=FA/FC
      R=FB/FC
      S=FB/FA
      P=S*(2.*XM*Q*(Q-R) - (B-A)*(R-1.))
      Q=(Q-1.)*(R-1.)*(S-1.)
C  ADJUST SIGNS
   60 IF (P .GT. 0.) Q=-Q
      P=ABS(P)
C  IS INTERPOLATION ACCEPTABLE?
```

```
            IF ((2.*P) .GE. (3.*XM*Q - ABS(TOL1*Q))) GOTO 70
            IF (P .GE. ABS(.5*E*Q)) GOTO 70
60          E=D
            D=P/Q
            GOTO 80
      C  BISECTION
         70 D=XM
65          E=D
      C  COMPLETE STEP
         80 A=B
            FA=FB
            IF (ABS(D) .GT. TOL1) B=B+D
70          IF (ABS(D) .LE. TOL1) B=B+SIGN(TOL1,XM)
            FB=F(B)
            IF ((FB*(FC/ABS(FC))) .GT. 0.) GOTO 20
            GOTO 30
      C  DONE
75       90 ZEROIN = B
            RETURN
            END

 1    CXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
            FUNCTION FLAGR(X,Y,XARG,IDEG,N)
      C
      C  INTERPOLATION ROUTINE FROM CARNAHAN, ET. AL
 5    C  ADVANCED NUMERICAL METHODS, P.31
      C  THIS ROUTINE COMPUTES AND EVALUATES THE LAGRANGE FORM OF THE
      C  INTERPOLATING POLYNOMIAL.  X AND Y SHOULD BE VECTORS OF LENGTH
      C  N CONTAINING THE INDEPENDENT VARIABLE (IN X) AND THE
      C  DEPENDENT VARIABLE (IN Y).  XARG IS WHERE THE INTERPOLATING
10    C  POLYNOMIAL IS TO BE EVALUATED.  IDEG IS THE DEGREE OF POLYNOMIAL
      C  TO BE USED.
      C
            REAL X(N),Y(N)
            FACTOR=1.0
15          MAX=1+IDEG
            DO 2 J=1,MAX
               IF (XARG .NE. X(J)) GOTO 2
               FLAGR=Y(J)
               RETURN
20       2     FACTOR = FACTOR*(XARG-X(J))
      C  EVALUATE INTERPOLATING POLYNOMIAL
            YEST=0.
            DO 5 I=1,MAX
               TERM=Y(I)*FACTOR/(XARG-X(I))
25             DO 4 J=1,MAX
         4        IF (I .NE. J) TERM=TERM/(X(I)-X(J))
         5     YEST = YEST + TERM
            FLAGR=YEST
            RETURN
30          END

 1    CXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
            FUNCTION F(X)
      C
      C  THIS FUNCTION IS A REWRITE OF THE DEFINING EQUATION FOR FF GIVEN
 5    C  VALUES FOR FBMAX, A, KO AND AMOL (MOLARITY).
      C
            COMMON A,AMOL,FBM,OK
            F = (OK * X) ** A * (FBM - AMOL * X) - AMOL * X
            RETURN
10          END
```

```
PIPERONYL BUTOXIDE 36 DAYS 147- 94      00
33.525      .0272      .02
3 1.
   1.E-09.2179      1.236
 3.33E-09.1509      1.533
  9.9E-09.07927     2.262
3 3.
   1.E-09.35917     .8150
 3.33E-09.3248      .8521
  9.9E-09.2341      1.094
3 10.
   1.E-09.4232      .6624
 3.33E-09.4231      .6491
  9.9E-09.3992      .7031
PIPERONYL BUTOXIDE 36 DAYS 147- 94      -1
33.525      .0272      .02
3 1.
   1.E-09.2179      1.236
 3.33E-09.1509      1.533
  9.9E-09.07927     2.262
3 3.
   1.E-09.35917     .8150
 3.33E-09.3248      .8521
  9.9E-09.2341      1.094
3 10.
   1.E-09.4232      .6624
 3.33E-09.4231      .6491
  9.9E-09.3992      .7031
.2034 E-076.5    E+00.84    E+00.7673 E+090.45    E+0
.2034 E-080.0    E+00.84    E-01.7673 E+080.0     E+00
THIS CARD ENDS THE RUN BECAUSE OF +1    +1
```

PIPERONYL BUTOXIDE 36 DAYS 147- 94

WITH 3 ANTIBODIES PF = .0272 QF= 3.5250

THE FIRST TERM IS P' FOR DATA SET 1	FIRST TERM IS Q' FOR DATA SET 1
.253335	1.10236
.217900	1.23600
.150900	1.53300
.079270	2.26200

THE FIRST TERM IS P' FOR DATA SET 2	FIRST TERM IS Q' FOR DATA SET 2
.374275	.80690
.359170	.81503
.324800	.85210
.284100	1.09400

THE FIRST TERM IS P' FOR DATA SET 3	FIRST TERM IS Q' FOR DATA SET 3
.421898	.67332
.423200	.66240
.423100	.64910
.399200	.70310

PB = .46635
SLOPE= -.91675
STANDARD ERROR = .01722

QB = .6204
SLOPE= .19962
STANDARD ERROR = .00711

FIRST APPROXIMATIONS
A = .840000
KD = .7673E+09
FBMAX = .2034E-07

LEAST SQUARES FIT

XI-SQUARED = .14121276E+01

FBMAX =	.7247E-08	+/-	.1271E-09
QF/QB =	8.8262	+/-	.8653E-01
A =	.765187	+/-	.3938E-02
KD =	.6091E+09	+/-	.4796E+07
PB =	.451781	+/-	.2915E-02
PF =	.027200	+/-	0.

ANTIBODY	MOLARITY	P OBSERVED	P CALCULATED
.10000000E+01	.10000000E-08	.217900	.217155
	.33300000E-08	.150900	.145685
	.99000000E-08	.079270	.071936
.30000000E+01	.10000000E-08	.359170	.360866
	.33300000E-08	.324800	.319260
	.99000000E-08	.234100	.238807
.10000000E+02	.10000000E-08	.423200	.429227
	.33300000E-08	.423100	.419469
	.99000000E-08	.399200	.402756

PIPERONYL BUTOXIDE 36 DAYS 147- 94 -1

WITH 3 ANTIBODIES PF = 3.5250 OF= .0272
LEAST SQUARES FIT

XI-SQUARED = .11947542E+01

FBMAX =	.6738E-08	+/-	.1271E-09
QF/QB =	6.6000	+/-	0.
A =	.774250	+/-	.5250E-02
KD =	.5131E+09	+/-	.4796E+07
PB =	.450000	+/-	0.
PF =	.027200	+/-	0.

ANTIBODY	MOLARITY	P OBSERVED	P CALCULATED
.10000000E+01			
	.10000000E-08	.217900	.216044
	.33300000E-08	.150900	.147699
	.99000000E-08	.079270	.076514
.30000000E+01			
	.10000000E-08	.359170	.357875
	.33300000E-08	.324800	.317228
	.99000000E-08	.234100	.237310
.10000000E+02			
	.10000000E-08	.423200	.426675
	.33300000E-08	.423100	.416910
	.99000000E-08	.399200	.400521